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Differentiation of *Agaricus* species and other homobasidiomycetes based on volatile production patterns using an electronic nose system

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Comparisons of the qualitative volatile production patterns between seven species of *Agaricus*, and between two of *Volvariella* and *Pleurotus* and one *Coprinus* species when grown at 25 °C on agar media for 14 d were made. There was good reproducibility between the volatile production patterns of the same species using an electronic nose unit with a 14 conducting sensor polymer array. Principle Component Analysis (PCA) showed that it was possible to discriminate between five of the seven *Agaricus* species, but that some overlap occurred between the others. Cluster analysis showed that there was also overlap between some species with the tropical collection of *A. bitorquis* separating out from the others. The volatile production profile of the commercial *A. bisporus* was close to that of a wild species, *A. campestris*. *A. bisporus* could be readily differentiated from other non-*Agaricus* species. This study demonstrates the potential for using electronic nose systems to rapidly differentiate mycelial cultures of homobasidiomycete mushrooms.

INTRODUCTION

There has recently been interest in rapid methods for the differentiation between related fungal species using a range of techniques including molecular methods, isoenzyme profiles, hydrolytic production profiles, and more recently volatile production patterns (Schnurer, Olsson & Borjesson 1999, Magan & Evans 2000).

Despite the importance of odour in the identification of homobasidiomycete mushrooms, no attempt has been made to detect and differentiate volatile production patterns of closely related species. Indeed Cappelli (1984) suggested that the smell of one species may vary according to the weather. Often it is necessary to bruise or crush the flesh in order to detect such odours. Table 1 shows the descriptors currently used to distinguish different homobasidiomycetes (Cappelli 1984, Jordan 1995). Electronic nose (e-nose) systems could provide a more definitive, less subjective method to define mushroom odours and studies like those described here could be used to provide benchmark odour values for a range of homobasidiomycetes, especially mycelial cultures, including *Agaricus*, to support taxonomic definitions.

Recent studies with spoilage fungi suggest the possibility of early detection of volatile production patterns using odour mapping devices which could enable detection of moulds contaminating food raw materials (Schnurer, Olsson & Borjesson 1999, Magan & Evans 2000). Indeed, developments in this technology suggest potential for rapid differentiation between species based on qualitative volatile production patterns. For example, Keshri *et al.* (1998, 2001) showed that spoilage fungi could be differentiated within 48 h of spore germination, both in agar media and in food matrices; Gibson *et al.* (1997) could distinguish between bacteria, and Magan, Pavlou & Chrysanthakis (2001) between spoilage bacteria and yeasts in milk media. To our knowledge no studies have been carried out to try and differentiate fresh mushrooms or mycelial cultures, although some freeze dried cultures have been examined (Breheret *et al.* 1995). Mushroom sporophores are more odiferous than mycelial cultures, and if the technology can discriminate between such mycelia, then it should prove even more effective using the mushrooms themselves.

The objective of this study was to evaluate the potential of an e-nose system for the differentiation of homobasidiomycete mushroom species based on mycelial cultures. In this paper we describe the qualitative patterns of volatile production for seven different species

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Table 1. Homobasidiomycete mushroom species used in electronic nose comparison; authorities, classification and description of odours.

Species	Classification[a]	Odour descriptions[b]
Agaricus arvensis	*Agaricaceae*, *Flavescentes* sect. *Arvenses*	Smell of almonds, strong of aniseed
A. bisporus	*Agaricaceae*, *Rubescentes* sect. *Bitorques*	Smell pleasant, marked, strong of mushroom
A. bitorquis	*Agaricaceae*, *Rubescentes* sect. *Bitorques*	Smell marked, agreeable, not distinctive
A. campestris	*Agaricaceae*, *Rubescentes* sect. *Agaricus*	Smell agreeable, not of almonds
A. nivescens	*Agaricaceae*, *Flavescentes* sect. *Arvenses*	Smell of almonds
A. maleolens	*Agaricaceae*, *Rubescentes* sect. *Bitorques*	Slightly fishy
A. subfloccosus	*Agaricaceae*, *Rubescentes* sect. *Sanguinolenti*	Smell somewhat unpleasant
Coprinus cinereus	*Coprinaceae*, *Laatuli* sect. *Lagopus*	Not distinctive
Pleurotus ostreatus	*Lentinaceae*	Not distinctive
P. sajor-caju	*Lentinaceae*	No description
Volvariella bombycina	*Pluteaceae*	Strong, pleasantly fungoid
V. volvacea	*Pluteaceae*	No description

[a] Sections in the genus *Agaricus* based on Cappelli (1984).
[b] From Cappelli (1984) and (or) Jordan (1995).

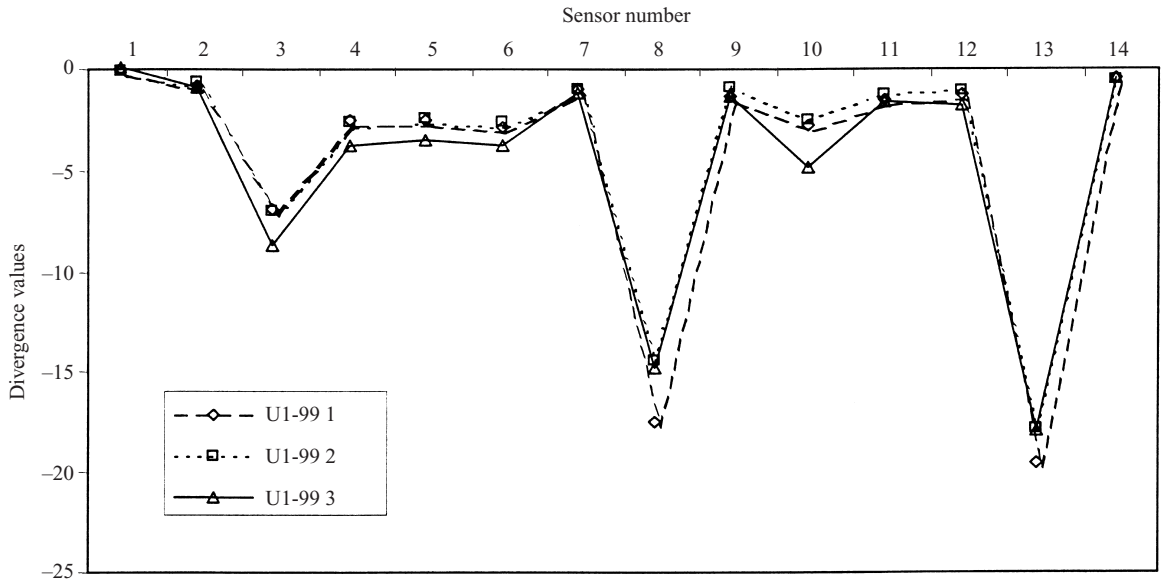

Fig. 1. Comparison of divergence response of 14 sensor conducting polymer array for randomised replicates of mycelial cultures of *Agaricus bisporus* (U1-99) after 14 d incubation at 25 °C.

of *Agaricus*, and for four other species from the diverse genera *Volvariella*, *Pleurotus* and *Coprinus*.

MATERIALS AND METHODS

Fungal species used in this study

Species within *Agaricus* are separated into two large groups, *Rubescentes* and *Flavescentes*, based on macrochemical reactions to the Schaffer colour assay. Within these, there are numerous sections and species (Cappelli 1984). In this study we have used collections that span both large groups and fall within four of the sections defined by Cappelli (1984).

The *Agaricus* species were: *Agaricus bisporus* U1-99, a wild strain W7 originally identified as *A. macrosporus* (Elliott 1978) but recently reclassified as *A. maleoleons* (Challen & Noble, unpubl.), *A. nivescens* W6I (Calvo-Bado *et al.* 2000), *A. arvensis* 93-7 (Calvo-Bado *et al.* 2000), *A. campestris* W1S, *A. bitorquis* W20 (Pahil, Smith & Elliott 1991) and *A. subfloccosus* W4II (Smith & Love 1995). The other species examined were *Volvariella bombycina* R83 (Elliott & Challen 1985), *V. volvacea* R171, *Pleurotus sajor-caju* R90, *Pleurotus ostreatus* R155 and *Coprinus cinereus* CcH9 (Challen *et al.* 1994). All strains were recovered from and are preserved in the HRI liquid nitrogen culture collection (Challen & Elliott 1986).

Mycelial cultures were grown up on malt peptone medium (malt extract, Oxoid L39 20 g l^{-1}, mycological peptone, Oxoid L40, 5 g l^{-1}) and 1.2% wt vol^{-1} agar (Oxoid Technical No. 3, L13). The agar medium was poured into 9 cm Petri plates and they were inoculated in three places on each plate with 4 mm diam agar plugs taken with a cork borer from the growing margin of colonies on the same medium for 14 d. Four replicate plates of each species were incubated at 25 °C for 14–21 d prior to e-nose measurements being carried out. All experiments were repeated once.

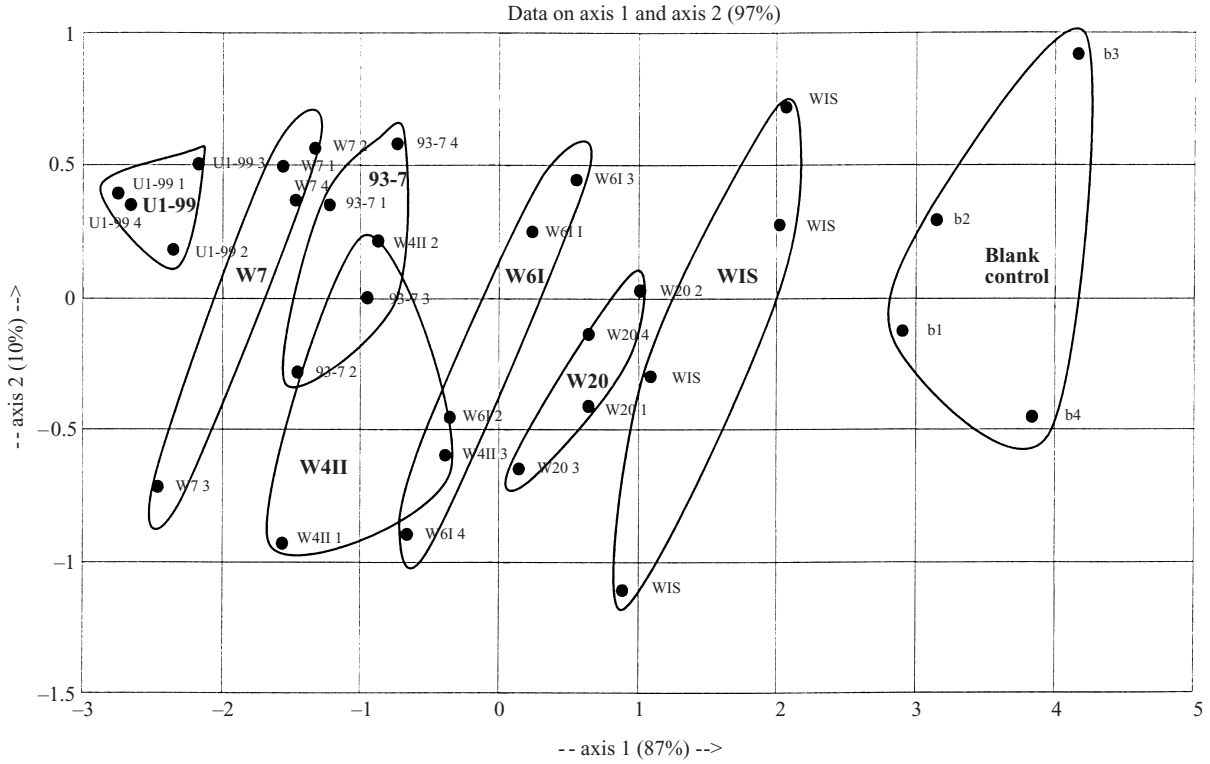

Fig. 2. Principle component analysis (PCA1, PCA2) of the data for all *Agaricus* species after 14 d incubation showing the groupings for the control blanks and all the species. PCA based on divergence data of all 14 sensors. Key to species: U1-99, *A. bisporus*; W7, *A. maleolens*; 93-7, *A. arvensis*; W4II, *A. subfloccosus*; W6I, *A. nivescens*; W20, *A. bitorquis*; W1S, *A. campestris*.

Sampling with electronic nose and data analyses

Individual plates were placed in a sampling bag (500 ml) and filled with filter sterilised air and sealed (Keshri & Magan 2001). After 2 h equilibration at 25 °, the headspace of each bag was sampled in a randomised fashion using a Bloodhound BH114 e-nose unit (Bloodhound Sensors, Leeds). This system uses 14 conducting polymer sensors which work best under ambient conditions. To minimise the influence of relative humidity all samples were prefiltered prior to entering the e-nose unit. The interaction of volatile compounds and the conducting polymer surface produces a change in resistance which can be amplified and analysed through the e-nose software package system. The flow rate over the sensors was fixed at 4 ml min^{-1} to generate the sensor baselines. Four sensor parameters can be studied with this system: adsorption (maximum rate of change of resistance), desorption (maximum negative rate of change of resistance), divergence (maximum step response) and area (area under the actual sensor curve). The divergence response was primarily used for this study, and the sampling profile was set at 15 s of adsorption and 22 s of desorption. This has been detailed elsewhere (Pavlou *et al.* 2000).

Normalised e-nose data were analysed using the programme xlStat (Microsoft Excel add-in program). Principle Component Analysis (PCA) and Cluster Analysis (CA) techniques were applied to differentiate and classify the fungal species. To carry out analyses the response from all the sensors were used. Cluster analysis was based on the Malahanobi's squared distance between groups obtained in the discriminant analysis at the 95% confidence limit.

RESULTS AND DISCUSSION

Fig. 1 shows the divergence data for the volatile profiles for three replicates of a single treatment (*Agaricus bisporus* U1-99) for all 14 sensors. The between replicate standard error was small, with an overall average of $<10\%$. The PCA (axis 1 and 2) accounts for 97% of the data for the *Agaricus* species and indicates the groupings obtained based on the volatile production profiles (Fig. 2). This also shows that there was differentiation between the uninoculated blank medium alone (control), and the *A. bisporus* (U1-99) strain at the two ends of the spectrum. Overall, PCA differentiation between the *Agaricus* species was robust, except for *A. subfloccus* (W4II) which overlapped with *A. arvensis* (93-7) and *A. nivescens* (W6I).

Examination of the data using Cluster Analysis (CA, Fig. 3) revealed that there were 3 different groups. *A. bisporus* (U1-99) and *A. campestris* (W1S) were grouped together, *A. bitorquis* (W20) was completely separated out, and the remaining species clustered from a third branch. Within the large cluster, two subgroups comprised *A. nivescens* (W6I) with *A. subfloccosus* (W4II) and *A. maleolens* (W7) with the control. Interestingly *A. avensis* (93-7) was differentiated from *A. nivescens* (W6I). These two species are inter-related

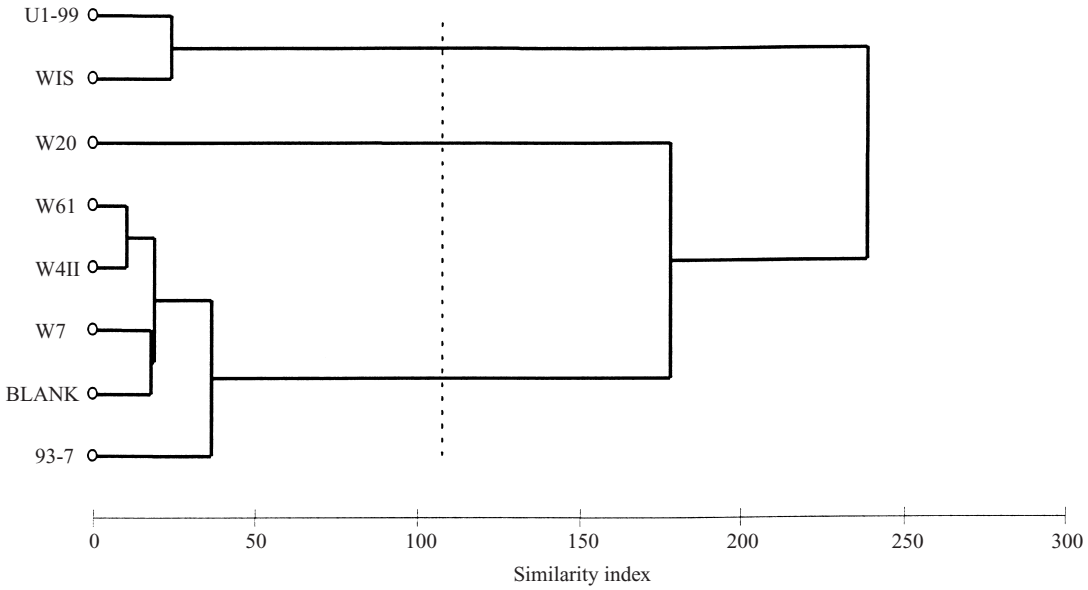

Fig. 3. Cluster analysis ($P=0.05$) of the data showing discrimination between the different species of *Agaricus*. The index indicates the relative distance between treatments. Key to species: see Fig. 1.

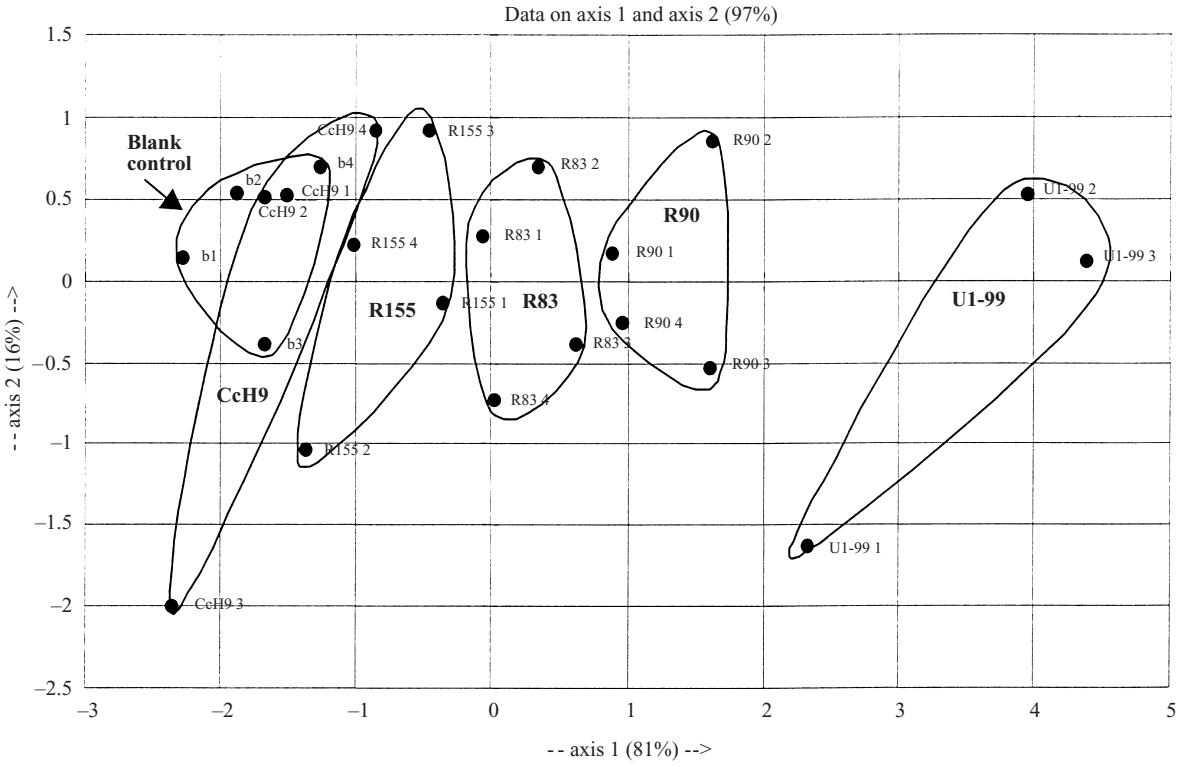

Fig. 4. Principle component analysis (PCA1, PCA2) of the divergence data of the 14 conducting polymer sensor array for comparisons between the genera *Agaricus*, *Pleurotus*, *Coprinus* and *Volvariella* after 14 d incubation. All treatments and replicates randomly sampled. Key to species: U1-99, *A. bisporus*; CcH9, *C. cinereus*; R155, *P. ostreatus*; R83, *V. bombycina*; R90, *P. sajor-caju.*

but can be distinguished by morphological, compatibility and DNA analyses (Calvo-Bado *et al.* 2000). Although some minor overlapping occurred between samples within the *Agaricus* genus, the differentiation between diverse homobasidiomycete genera was more obvious based on volatile production patterns. This suggests that e-nose may need further optimisation to detect subtle variations in volatile production patterns in species within a single genus.

Fig. 4 shows that when comparing the data for *A. bisporus* (U1-99) and species from other homobasidiomycete genera the PCA (axis 1 and 2) accounts

for 97% of the data. This shows that mycelial cultures of *A. bisporus* (U1-99) could be differentiated from *Volvariella*, *Pleurotus* and *Coprinus* species. There was very low volatile production by *C. cinereus* this resulted in an overlap with the blank control sample.

This study suggests that discriminating between genera may be readily achieved on the basis of volatile production profiles of mycelial cultures and shows that within *Agaricus* even some closely related species can be differentiated. It has been established that growth media and culture age affects type and concentration of volatiles produced (Schnurer, Olsson & Borjesson 1999, Magan & Evans 2000). Thus, better discrimination may be possible by optimising these criteria. There is only one reported investigation of an attempt to use e-nose systems to assess differences in mushroom odours. Freeze dried cultures of 9 wild species were examined with a laboratory e-nose system and GC. Both techniques differentiated the species into the same four groups. One of the mushroom species was clearly discriminated from the others (Breheret *et al.* 1995). This work was unfortunately not followed up with any studies on actively growing colonies or basidiomes.

Mushroom identification is still largely based on gross morphology. Recently molecular techniques such as the sequencing of rRNA genes are becoming increasingly pervasive in the resolution of fungal taxonomic groupings (Hibbett *et al.* 1997, Monocalvo *et al.* 2000). In *Agaricus*, ITS sequences have been used to differentiate species that are otherwise difficult to distinguish (Calvo-Bado *et al.* 2000). It is therefore appropriate to compare molecular and volatile profiling technologies with respect to their ability to discriminate species. In this study, the CA cluster groups were not always consistent with relationships determined from the molecular analysis. In terms of genetic identity, *A. arvensis* and *A. nivescens* are closely related, both fall within the section *Arvenses* and although different species, they are capable of limited interbreeding (Calvo-Bado *et al.* 2000). A more striking difference is the comparison between *A. bisporus* and *A. subfloccosus*. ITS sequencing and other genetic analysis indicate that these two species are very closely related (Challen, Kerrigan & Callac, unpubl.) while in this study, *A. bisporus* and *A. subfloccosus* fall into different CA clusters. Odour cluster groups can therefore differ from those determined by ITS sequencing and, our study shows that a multi-array e-nose can prove more discriminatory than molecular markers limited to short specific sequences. E-nose technology is a fast moving field and there are continuing improvements in both the sensitivity of the sensor arrays and the range of detectable volatiles. Improved discrimination between species is highly probable and this opens the possibility for database development to assist with rapid identification of such fungal species.

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